PAROXYSMAL HEMOGLOBINURIA.

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As a clinical entity, paroxysmal hemoglobinuria was first recognized and described by Dressler in 1854. In spite of the many clinical studies and the amount of experimental work on isolated cases, however, no satisfactory hypothesis was advanced to explain the pathology of this rare and curious disease until 1904, when Donath and Landsteiner[1] contributed their first paper to the subject. They attributed the attack characteristic of the disease to a specific hemolyzing substance in the blood of these patients, and they demonstrated its presence by experiments *in vitro*. Eason[2] working independently, had found this same hemolyzing substance in a case under his care. Since then a number of observations have been made by different investigators, all of whom confirm the presence of the autolysin; but with regard to the definite mode of action of this substance on red blood corpuscles there is even now no unanimity of opinion. The case here reported was studied with a view to clearing up, if possible, some of the disputed points.

History. E. P., aged thirty-eight years, porter, colored, single, was admitted to the hospital November 10, 1911.

Family History. Father, four brothers, and four sisters dead; cause unknown. Mother and one sister living and well. The sister resides in New York City and, so far as known, has never had an attack of hemoglobinuria.

Personal History. Patient was born in the West Indies, where he lived until eleven years ago, when he came to New York City. His habits are good; alcohol and tobacco in moderation.

Previous History. An indefinite history of a short, mild attack, called malaria, once before coming to New York. Veinereal disease denied, and no symptoms elicited suggesting an acquired infection.

Present History. Nine years ago, having been in New York City two years, the patient had an attack which came on suddenly in November, 1902, while he was working and apparently in excellent health. This attack consisted of a severe shaking chill, with fever, followed by the passing of black urine. In twelve hours he was as well as usual, and the urine looked normal. One week later he had a similar experience. Beginning one month later, January, 1903, he had attacks in rapid succession, and was in a hospital for ten weeks, without improvement, the paroxysms continuing at intervals throughout the summer and up to September, 1903, when they ceased spontaneously, and he was perfectly well for two years. Since September, 1905, the paroxysms have recurred at irregular intervals, for the most part in the cold season, but occasionally in summer. He found that washing his hands in cold water would induce an attack, and he says the summer attacks always followed chilling in a draught after hard work. All of the attacks since January, 1903, have been associated with severe, sharp, lancinating pain in the lumbar region, especially the right. There is also numbness and tingling of the extremities, which subsides with the attack.

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[1] Münch. med. Woch., 1904, li, 1590; Zeit. f. klin. Med., 1906, lviii, 173.
[2] Edinburgh Med. Jour., 1906, xix, 43; Jour. Path. and Bact., 1906–7, xi, 167.

Physical Examination. The patient is a well-built, rather small negro. He is absolutely normal, with the exception that on abdominal examination a smooth rounded mass is found in the upper quadrant on each side, taken to be the kidney, moderately enlarged, slightly movable, and not tender.

Cystoscopy: December 19, 1911, by Dr. E. L. Keyes, Jr. Bladder normal, mouths of the ureters normal, bloody urine from both ureters.

Radiograph: "Does not show calculus in either urinary tract."

Urine Examination: During an interval the urine is clear, of normal specific gravity, with no albumin. An occasional cast is found, but no pus cells and no red blood cells. During an attack the urine is dark reddish brown, containing much coagulable proteid, few leukocytes, and an occasional red blood cell. Chemical test for blood, positive.

Blood Examination: Without special reference to the attacks the blood shows: Leukocytes, 4400 to 7200; polymorphonuclears, 62 per cent. to 65 per cent.; hemoglobin (Dare), 62 per cent. to 65 per cent. (November to December, 1911), 72 per cent. (March 16, 1912).

Red blood cells, 2,700,000 (November 29, 1911), 3,600,000 (February 14, 1912), 4,800,000 (March 16, 1912). Coagulation time, eight to nine minutes (normal). Blood plates, 500,000. Malarial parasites, absent.

Wassermann Reaction: Positive, December 4, 1911.

Salvarsan: 0.5 gm., given intravenously, February 19, 1912.

Wassermann Reaction: Positive, March 25, 1912 (by Dr. L'Esperance).

Salvarsan: 0.4 gm., given intravenously, April 7, 1912.

Wassermann Reaction: Positive, April 15, 1912 (by Dr. Noguchi).

Luetin Test: Positive April 15, 1912 (by Dr. Noguchi).

Salvarsan: 0.6 gm., given intravenously, April 29, 1912.

Wasserman Reaction: Positive, May 30, 1912.

On January 5, 1912, the Ehrlich experiment was done. A tourniquet was placed about the left arm above the elbow, and the forearm was immersed in ice water for five minutes. The arm was then taken out and dried, and blood was drawn from a finger into a test-tube containing a small amount of 0.8 per cent. NaCl solution. This was warmed and centrifuged. Hemolysis was present. As a control, blood was taken from a finger on the right hand, which was not immersed, before the tourniquet was removed from the left arm. This was also put in a test-tube containing a small amount of salt solution and warmed. Hemolysis was absent. During the immersion the patient complained of intense lumbar pain, with tingling of all the extremities. Within a half hour after the experiment the patient had a severe shaking chill, with rise of temperature to 102° F. The first urine voided was dark reddish brown. Urine voided three hours later was also dark brown, but the urine voided six hours after the experiment was perfectly clear amber colored.

We have then to do with a perfectly typical case of paroxysmal hemoglobinuria in a man without history and with none of the stigmata of congenital or acquired syphilis, but with a positive Wassermann reaction and positive luetin test.

The particular point of interest in this pathological condition is the hemolysin, which will be considered under the following headings: (1) Demonstration of the autohemolysin; (2) absorption of the auto-antibody from inactive serum; (3) dissociation of corpuscle antibody combination; (4) absorption of complement from active serum; (5) action of complement on red blood cells sensitized with inactive hemolytic serum; (6) mode of action of the autohemolysin; (7) etiology of paroxysmal hemoglobinuria.

The preparations used in the work here reported are designated as follows: (1) "P. Ser." The serum of the hemoglobinuric patient obtained by centrifuging defibrinated blood while still warm. (2) "P. Ex. Ser." Defibrinated blood of the same case was subjected to 0° C. for one hour, then centrifuged quickly in the cold and the serum removed. (3) "P. Ina. Ser." Some of the serum obtained in (1) was inactivated at 55° C. for twenty minutes. (4) "N. H. S." Normal human serum obtained from defibrinated blood. (5) "N. Ex. Ser." Defibrinated blood, same as (4), at 0° C. for one hour, centrifuged quickly in the cold and the serum removed. (6) "G. P. Ser." Guinea-pig serum from defibrinated blood. (7) 10 per cent. "P. Blood." From the hemoglobinuric case. (8) 10 per cent. "N. H. Blood." Normal human blood from the same case as the serum in (4). (9) 10 per cent. sensitized sheep's blood.

For these suspensions (7 to 9) 5 c.c. of the defibrinated blood were washed three times with 0.8 per cent. warm NaCl solution and made to 50 c.c., with 0.8 per cent. NaCl solution. All preparations were freshly made on the day in which the experiments were done. In every instance at least seven days had intervened since an attack. These experiments are confirmatory of a number of earlier experiments carried out on this case, in which hemolysis was shown, not only for his own but other human corpuscles. In incubating for hemolysis it was found that after icing, if the tubes were warmed over a free flame carefully, one-half hour in the incubator was sufficient for maximum hemolysis. This technique was employed in all my cold-warm experiments.

1. Demonstration of the Autohemolysin. By subjecting oxalated blood of a hemoglobinuric patient or the serum and a corpuscle suspension of the same blood to a low temperature, then incubating at 37° C., Donath and Landsteiner obtained hemolysis "in vitro." No hemolysis took place on prolonged exposure to the cold only nor on prolonged incubation at 37° C. only. This so-called "cold-warm" experiment has given positive results in the hands of all other observers, although Meyer and Emmerich[3] were successful only thirty times in a total of sixty-nine experiments on four patients, and Kumagai and Inoue[4] were compelled to modify the original technique to get satisfactory results. In the twelve separate times that the case here reported was examined, positive results were always obtained by the original Donath-Landsteiner method.

Table I.—Demonstration of Autohemolysin.

A.

		Lysis.
0.200 cc P. Ser. + 0.25 cc 10% P. Blood	0° C. ½ hr. Warmed 37° C. ½ hr.	Nearly complete
0.100 cc P. Ser. + 0.25 cc 10% P. Blood		Partial
0.050 cc P. Ser. + 0.25 cc 10% P. Blood		Slight
0.025 cc P. Ser. + 0.25 cc 10% P. Blood		0
Control: 0.2 cc P. Ser. + 0.25 cc 10% P. Blood	37° C. 1 hr.	0
Control: 0.2 cc P. Ser. + 0.25 cc 10% P. Blood	0° C. 1 hr.	0

B.

		Lysis.
0.2000 cc P. Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood	0° C. ½ hr. Warmed 37° C. ½ hr.	Complete
0.1000 cc P. Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood		Nearly complete
0.0500 cc P. Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood		Strong
0.0250 cc P. Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood		Trace
0.0125 cc P. Ser. + 0.3 cc N. H. S. + 0.25 cc 10 %P. Blood		0

C.

		Lysis.		Lysis.
0.3 cc N. H. S. + 0.25 cc 10% P. Blood	37° C. 1 hr.	0	Warmed 37° C. ½ hr.	0
0.3 cc N. H. S. + 0.25 cc 10% N. H. Blood		0		0

To demonstrate the autolysin, the experiments given in Table I were done. The results obtained show the characteristic hemolytic action with the "cold-warm" experiment, while the controls, that is, icing alone and incubating alone, are negative. Comparison of A with B shows a deficiency of complement in the "P. Ser." to completely activate all the antibody present, for the addition of complement by "N. H. S." in B shows a smaller complete lytic dose than in A and establishes 0.2 c.c. as the minimum complete lytic dose of the "P. Ser." for the amount of corpuscles used, in the presence of sufficient complement. C shows the absence of autolysin in "N. H. S." and the absence of an isolysin for "P. Blood," and this serum can therefore be used as complement.

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[3] Deut. Arch. f. klin. Med., 1909, xcvi, 287.

[4] Deut. med. Woch., 1912, No. 8, p. 361.

2. ABSORPTION OF ANTIBODY FROM INACTIVE SERUM. Donath and Landsteiner, and Eason also, in their first papers demonstrated the hemolysin concerned in paroxysmal hemoglobinuria as an antibody-complement complex. In their later work reactivating serum was added to the inactive hemoglobinuric serum and corpuscles and hemolysis resulted with the "cold-warm" experiment. These earlier experiments do not, however, deal with the point in question. There has arisen some divergence of opinion among later workers as to the capacity of the red blood cell to absorb antibody from inactive serum on exposure to the cold. Meyer and Emmerich[5] and Hoover and Stone[6] demonstrated that such absorption could take place, but Moss[7] could not confirm their results, and concluded that "union between auto-amboceptor and corpuscle takes place only at a low temperature, in the presence of complement."

This question can best be approached by a quantitative study of the lytic action of the inactive serum before and after exposure to corpuscles in the cold. The experiments were done as follows:

Experiment A. "P. Ina. Ser." was titrated by mixing with 0.3 c.c. normal human serum (complement) and then 0.25 c.c. 10 per cent. "P. Blood" added—incubated at 0° C. one-half hour and then warmed and incubated at 37° C. one-half hour.

Experiment B. Six complete lytic amounts (0.2 c.c. equals a complete lytic dose) of "P. Ina, Ser." were iced for one-half hour, with the sediment of four times six units (0.25 c.c. equals one unit) of 10 per cent. "P. Blood"—centrifuged in the cold and the serum removed for titration, as in A, by mixing with 0.3 c.c. normal human serum and then adding 0.25 c.c. 10 per cent. "P. Blood." Incubated at 0° C. for one-half hour, warmed, incubated at 37° C. one-half hour. The results are shown in Table II.

TABLE II.—Absorption of Antibody from Inactive Serum.

In each tube 0.3 cc N. H. S. + 0.25 cc 10% P. Blood

	A.—P. ina. Ser.	B.—P. ina. Ser. after 0° C. ½ hr. with Sed. P. Blood
	Hemolysis after 0° C. ½ hr.—warmed—37° C. ½ hr.	
0.4000 cc	Complete	Partial
0.2000 cc	Complete	Slight
0.1000 cc	Nearly complete	0
0.0500 cc	Very strong	0
0 0250 cc	Slight	
0.0125 cc	0	

Control: 0.4 cc P. ina. Ser. + 0.25 cc 10% P. Blood + 0° C. ½ hr. + warm + 37° C. ½ hr. = hemolysis, 0

In A the inactive serum was reactivated and gave complete hemolysis, with 0.2 c.c., the same minimum complete lytic dose as active serum plus complement, as shown in Table I—B. In B, as described here, the serum was exposed to "red blood cells" in the cold when no complement was present, as shown by the absence of hemolysis in the control. The serum was removed while still cold, and on titration, after adding complement, we find only "partial" hemolysis with 0.4 c.c. It is evident, therefore, that antibody was removed, and from the result about 85 per cent was absorbed by the corpuscles, in the absence of complement, on exposure to cold.

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[5] Loc. cit.

[6] Arch. Int. Med., 1908, iii, 392.

[7] Johns Hopkins Hosp. Bull., 1911, xxii, 238.

3. Dissociation of Corpuscle-antibody Combination. In order further to understand the behavior of the autolysin it is necessary to determine whether or not there is ever a dissociation of antibody from corpuscle on subsequent elevation of temperature after they have been united by exposure in the cold. Meyer and Emmerich state that such dissociation does occur, and they were able to remove antibody from the sensitized red blood cells by washing them with warm NaCl solution. To determine this the following tests were made:

Experiment A. Two and one-half complete lytic doses of the "P. Ina. Ser." were added to the sediment of ten times two and one-half units of 10 per cent. "P. Blood," iced for one-half hour, centrifuged immediately in the cold, and the serum decanted and used for titration, as shown in Table III—A.

Experiment B. Two and one-half complete lytic doses of "P. Ina. Ser." were added to the sediment of ten times two and one-half units of 10 per cent. "P. Blood," iced for one-half hour. It was then warmed and incubated at 37° C. for one-half hour, centrifuged, and the decanted serum titrated just as in A. The results are shown in Table III—B.

Experiment C. This gives the titration of the "Ina. Ser." for this day, and shows 0.4 c.cm. as the minimum complete lytic dose of this serum. See Table III—C.

TABLE III.

A.—Titration of P. ina. Ser. after 0° C. ½ hr. with red blood cell and decant at once.

		Lysis.
0.4 cc decant Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood	0° C. ½ hr.	Faint trace
0.2 cc decant Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood	Warmed	0
0.1 cc decant Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood	37° C. ½ hr.	0

B.—Titration of P. ina. Ser. after 0° C. ½ hr., then 37° C. ½ hr. with red blood cell then decant.

		Lysis.
0.40 cc decant Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood	0° C. ½ hr. Warmed 37° C. ½ hr.	Partial
0.20 cc decant Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood		Slight
0.10 cc decant Ser. + 0.3 cc N. H. S. + 0 25 cc 10% P. Blood		Trace
0.05 cc decant Ser. + 0.3 cc N. H. S. + 0 25 cc 10% P. Blood		Faint trace

C.—Titration of P. ina. Ser.

		Lysis.
0.400 cc P. ina. Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood	0° C. ½ hr. Warmed 37° C. ½ hr.	Complete
0.200 cc P. ina. Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood		Very strong
0.100 cc P. ina. Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood		Strong
0.050 cc P. ina. Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood		Slight
0.025 cc P. ina. Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood		0
Control: 0.4 cc P. ina. Ser. + 0.25 cc 10% P. Blood		0

In A the inactive serum was exposed to a large body of corpuscles in the cold, and the serum was removed while still cold. As we have already seen, antibody is removed from serum so treated. In this experiment, when the decanted serum is reactivated and put through the "cold-warm" test, only a faint trace of hemolysis results with 0.4 c.c., and we may, therefore, conclude that practically all the antibody has been absorbed by the corpuscles. In B the same amount of inactive serum was exposed to the same amount of corpuscles for the same length of time. It is fair to assume that the same amount of antibody was removed from the serum by the corpuscles. But this inactive serum was left with its corpuscles for one-half hour in the incubator before separating it. On decanting and titrating it just as in A we find that 0.4 c.c. gives "partial" hemolysis and 0.1 c.c. gives a "trace." We have, therefore, demonstrated that antibody is dissociated from corpuscle at body temperature, for in this experiment about 25 per cent. of that absorbed was liberated. The corpuscle-antibody combination evidently is not an absolutely stable one. The difficulty of absorbing all the antibody from the serum, no matter how great the excess of corpuscles, is shown in the experiment already given, as well as in some of those that follow. This fact would suggest that an equilibrium is established on the part of antibody between the corpuscles, whose absorptive power is greatly enhanced by the action of cold, and the serum. This has its analogy in chemistry in the behavior of two immiscible solvents toward a solute. This fact, however, does not permit of an interpretation of the behavior of antibody on a definite and purely chemical basis in contract to a biological one. That antibody would be returned in its entirety to the serum on more prolonged incubation is conceivable, but its demonstration is not essential for the purposes of discussion here.

4. Absorption of Complement from Active Serum. Apparently biased by the well-known behavior of complement and antibody in ordinary immune sera, that is, the capacity of an antibody to unite with its antigen in the cold, while complement remains free, Donath and Landsteiner maintain the view that cold is necessary only for the union of antibody and corpuscle and that complement unites at the higher temperature, with resulting hemolysis. They have, however, no experiments bearing directly upon this point. Eason concurs with this idea, but likewise without proof. Hoover and Stone were unable to get any hemolysis unless there was an exposure of corpuscle and inactive serum to the cold after complement was added, and they first suggested that cold was necessary both for the union of complement and antibody, as well as antibody and corpuscle. Meyer and Emmerich contend that cold is not necessary for antibody complement combination. They merely state their results and give no detailed protocols of the work upon which their conclusions rest. Moss states that "the complement

does not enter into the combination, or at least not permanently, at this temperature," for he was able to demonstrate complementary action in the decanted serum after active serum was chilled with corpuscles. His work may be criticised on the ground that it was not quantitative.

Whether complement does or does not enter into combination on exposure to the cold can best be determined by a titration of complement in active serum before and after the serum is exposed to corpuscles in the cold. The experiments are given in Table IV. In Table IV—A the titrations of complement were made against 10 per cent. sensitized sheep's blood. These sheep corpuscles were sensitized by the addition of antibody in the form of inactive serum of rabbit that was immune to sheep red cells. In the first column we see that "P. Ser." that is, active serum before exposure to corpuscles in the cold, is able to act as complement, and 0.05 c.c. is enough to induce complete hemolysis. In the second column, "P. Ex, Ser." is used. This, as stated above, is serum derived from the original defibrinated blood after it was iced for one hour. 0.4 c.c. of this serum has no complementary action, and induces no hemolysis whatever. That it cannot be said that the treatment destroyed complement, a control was done with normal human serum under absolutely identical conditions, and here we see (columns 4 and 5) that the exposure to corpuscles in the cold did not decrease the complement, but actually increased it by 100 per cent. This rather interesting and curious result has been obtained with several normal sera. The titer of "G. P. Ser." is given to show the degree of sensitization of the corpuscles used. "P. Ina. Ser." was also titrated against this hemolytic system to demonstrate its complete inactivation.

TABLE IV.

A.—Titration of Complement.

In each tube 0.25 cc 10% Sensitized Sheep Blood.

Amount of complement	P. Ser.	P. Ex. Ser.	P. ina. Ser.	N. H. S.	N. Ex. Ser.	G. P. Ser.
	Hemolysis after warming and incubating at 37° C. ½ hr.					
0.500000	......	..	0			
0.400000	......	0	0			
0.100000	Complete	..	..	Complete	Complete	
0.050000	Complete	..	..	Very strong	Complete	
0.025000	Partial	..	..	Slight	Very strong	
0.012500	Trace	..	..	Trace	Slight	Complete
0.006250	......	..	..	.....	.....	Complete
0.003125	......	..	..	.....	.....	Nearly complete

B.—Titration of P. Ex. Ser.

0.40 cc P. Ex. Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood	0° C. ½ hr. Warmed 37° C. ½ hr.	Very strong
0.20 cc P. Ex. Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood		Strong
0.10 cc P. Ex. Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood		Slight
0.05 cc P. Ex. Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood		0
Control: 0.4 cc P. Ex. Ser. + 0.25 cc 10% P. Blood		0

It is also necessary to demonstrate that complement is absent from the "P. Ex. Ser." as far as "P. Blood" is concerned. See Table IV—B. The control shows no hemolytic action of the "P. Ex. Ser." on "P. Blood." If we add complement ("N. H. S.") hemolysis results. This shows that the "P. Ex. Ser." contains antibody, about 15 per cent. of the original amount, and lack of hemolysis in the control must be ascribed to lack of complement. Here we have conclusive proof by two methods of the disappearance of complement from active serum, that is, its absorption from active serum by exposure to corpuscles in the cold. It is shown also that this disappearance of complement takes place only from that serum ("P. Ser.") containing a specific antibody, and not from normal serum. The evidence that complement unites with antibody only under the influence of cold will be given under Section 5.

5. ACTION OF COMPLEMENT ON RED BLOOD CELLS SENSITIZED WITH INACTIVE HEMOLYTIC SERUM. The previous experiments show that all the complement and 85 per cent. of the antibody was removed from the "P. Ex. Ser.," and must have been taken up by the corpuscles in the cold. The proof of this last assumption will be considered later. If complement is absorbed at one and the same time with antibody, can it or can it not unite with antibody subsequent to the union of the latter with corpuscles. And if it can unite subsequently, does this union take place in the warm or only under the influence of cold? The fact that complement is absorbed in the cold, which is quite contrary to the usual behavior of complement in immune sera, would certainly suggest that it must do so to act at all. The following experiments were done to determine the action of complement on corpuscles previously sensitized by exposure in ice to inactive hemolytic serum.

Experiment A. Four times two doses of the "P. Ina. Ser." (1.6 c.c.) and four units of 10 per cent. "P. Blood" (1 c.c.) at 0° C. one-half hour, centrifuged in the cold, decant the serum, and wash the corpuscles once with ice cold 0.8 per cent NaCl solution and suspend in 1 c.c. (original volume) of cold salt solution. These corpuscles, kept cold, were then used to titrate the "P. Ina, Ser." after it had been complemented with "N. H. S." The results are shown in Table V. The results obtained in the titration of the "P. Ina, Ser.." against unsensitized cells (Table II—A) are reproduced for comparison.

Experiment B. In this case a larger proportion of "P. Ina. Ser." was added and left in the cold for a longer period of time with corpuscles to sensitize them, if possible, more strongly. They were then used as in Experiment A: Five times four doses of "P. Ina. Ser." and five units of 10 per cent. "P. Blood" were put at 0° C. for one hour, centrifuged in the cold, washed once with ice-cold salt solution, and the corpuscles suspended in the original volume (1.25 c.c.) of ice-cold salt solution. The titration with these strongly sensitized cells is shown in Table V—B.

TABLE V.

A.—Titration of P. ina. Ser. using Mod. Sensitized 10% P. Blood.

		Lysis	Tab. II–A
0.40 cc P.ina.Ser.+0.3 cc N.H.S.+0.25 cc 10% Sens.P.Blood	0°C. ½ hr. Warmed 37°C. ½ hr.	Strong	Complete
0.20 cc P.ina.Ser.+0.3 cc N.H.S.+0.25 cc 10% Sens.P.Blood		Strong	Complete
0.10 cc P.ina.Ser.+0.3 cc N.H.S.+0.25 cc 10% Sens.P.Blood		Partial	Complete
0.05 cc P.ina.Ser.+0.3 cc N.H.S.+0.25 cc 10% Sens.P.Blood		Slight	Strong

B.—Titration of P. ina. Ser. using Strongly Sens. 10% P. Blood.

		Lysis.
0.40 cc P.ina.Ser.+0.3 cc N.H.S.+0.25 cc 10% Sens.P.Blood	0°C. ½ hr. Warmed 37°C. ½ hr.	Trace
0.20 cc P.ina.Ser.+0.3 cc N.H.S.+0.25 cc 10% Sens.P.Blood		Trace
0.10 cc P.ina.Ser.+0.3 cc N.H.S.+0.25 cc 10% Sens.P.Blood		Trace
0.05 cc P.ina.Ser.+0.3 cc N.H.S.+0.25 cc 10% Sens.P.Blood		Trace

In this experiment we have a demonstration of a very curious inhibiting action. That this is not due to any substance originally present in the inactive serum is evidenced by the fact that in Experiment II—A this same serum in combination with complement gave active hemolysis. The result must be due in some way to the condition of the red blood cells as a result of their previous treatment, and there are three possible hypotheses upon which it may be explained: (1) That complement must unite with antibody at the same time that antibody unites with corpuscle; (2) deflection of complement; (3) complementoid inhibition.

To determine which of these explanations suffices, the following experiments were done:

Experiment A. This gives the titration of the "P. Ina. Ser." for this day.

Experiment B. Five times four doses of "P. Ina. Ser." (0.2 c.c. equals one dose) plus five units of 10 per cent. "P. Blood," iced for one hour, centrifuged, serum decanted, and the corpuscles washed once with ice-cold salt solution and suspended in the original volume (1.25 c.c.) of ice-cold salt solution. The corpuscle suspensions were divided into five tubes, 0.25 c.c. to each tube, the first four kept ice-cold and treated as shown in Table VI—B. The fifth tube was carefully warmed over a free flame; complement was then added and the tube kept at 37° C. for one hour.

TABLE VI.

A.—Titration of P. ina. Ser.

		Lysis.
0.40 cc P. ina. Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood	0° C. ½ hr. Warmed 37° C. ½ hr.	Complete
0.20 cc P. ina. Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood		Complete
0.10 cc P. ina. Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood		Nearly comp.
0.05 cc P. ina. Ser. + 0.3 cc N. H. S. + 0.25 cc 10% P. Blood		Very strong
0.04 cc P. ina. Ser. .. .. .. + 0.25 cc 10% P. Blood		0
0.3 cc N. H. S. + 0.25 cc 10% P. Blood	37° C. 1 hr.	0

B.—Titration of P. ina. Ser. using Sensitized P. Blood.

		Lysis.
0.4 cc P. ina. Ser. + 0.3 cc N.H.S. + 0.25 cc 10% Sens. P. Blood	0° C. ½ hr. Warmed 37° C. ½ hr.	Very strong
0.2 cc P. ina. Ser. + 0.3 cc N.H.S. + 0.25 cc 10% Sens. P. Blood		Very strong
0.1 cc P. ina. Ser. + 0.3 cc N.H.S. + 0.25 cc 10% Sens. P. Blood		Very strong
0.3 cc N.H.S. + 0.25 cc 10% Sens. P. Blood		Very Strong
0.3 cc N.H.S. + 0.25 cc 10% Sens. P. Blood	37° C. 1 hr.	0

In this case, as in the former, there is definite inhibition of hemolysis, as shown by comparison of A with B, for with the sensitized corpuscles twice the complete lytic dose only gives "very strong" lysis. These corpuscles, as did those in V—B, all give the same amount of lysis irrespective of the amount of inactive serum added, and this is the case even in the fourth tube, where complement alone was added. Our first hypothesis them nust be discarded, for we have demonstrated by this fourth tube that complement can attach itself to antibody after antibody has been attached to corpuscle; but B—5 shows further that this union can take place only under the influence of cold, as already stated by Hoover and Stone, for if, after icing, tubes are carefully and quickly warmed over a free flame and complement added, as done here, the results are uniformly negative. This cannot be attributed to separation of antibody, for it has already been shown that this is rather reluctantly given off from the corpuscle on warming. Secondly, there is no ground for an explanation on the basis of "deflection of complement," so-called, for the less antibody added the less deflection possible, and the amount of hemolysis would be in inverse proportion to the amount of antibody added. This is not the case. The third hypothesis—compelmentoid inhibition—is an extremely interesting one. At the present day serologists, generally speaking, agree with Ehrlich's original conception that complement is composed of two fractions: the "endstück," or toxic part, which in hemolysis produces the lytic action, and a "mittelstück," by which the toxic portion is joined to the antibody. The "endstück" is thermolabile, and, therefore, absent in inactive serum, while the "mittelstück" is thermostable, and present in inactive serum. It has already been shown in this paper that the complement in serum of paroxysmal hemoglobinuria unites with antibody on exposure to corpuscle in the cold. There is, therefore, every reason to believe that complementoid will attach itself to antibody, and corpuscles when exposed to inactive serum take up antibody and antibody takes up complementoid in so far as this happens to be present, and will, therefore, block complement. That some hemolysis took place in the experiments can be attributed to the fact that all the antibody was not completely saturated with complementoid, for in none of the experiments on the serum of this case was there enough complement to completely activate all the antibody present. The question of complementoid in this serum has been studied further by a series of experiments, the protocols and discussion of which are reserved for a later paper; suffice it to say here that the presence of complementoid is shown.

6. Mode of Action of the Autohemolysin. To review briefly then we have seen that antibody is taken up from inactive serum in the cold, that is, is absorbed in the absence of complement; that this combination is not absolutely stable, for a certain amount is

dissociated on elevation of temperature; we have also seen that complement is absorbed from active serum, together with antibody, in the cold, that complement unites with antibody only under the influence of cold, and finally, that the red blood cells exposed to inactive serum in the cold are more or less resistant to hemolysis on account of the complementoid present in inactive serum, the degree of resistance depending on the amount of complementoid present. These facts warrant the following statement as to the mode of action of the hemolysin: Antibody unites with corpuscle and complement unites with antibody only under the influence of cold, the lytic action being manifested on subsequent elevation of temperature. They cannot unite in any other way.

In order further to sustain this theory, we must be able to interpret the result of various experiments by it.

Experiment 1. One complete lytic dose (0.2 c.c.) of "P. Ina. Ser." plus 0.25 c.c. 10 per cent. "P. Blood," iced for one-half hour. Centrifuged in the cold and save "decant" serum for the next experiment. The corpuscles were suspended in 0.45 c.c. of cold 0.8 per cent. NaCl solution and 0.3 c.c. "N. H. S." added and incubated at 0° C. for one-half hour, warmed, then incubated at 37° C. for one-half hour. Result, hemolysis slight.

Experiment 2. The "decant" serum was added to the sediment of 0.25 c.c. 10 per cent. "P. Blood," with 0.3 c.c. "N. H. S.," iced for one-half hour, incubated at 37° C. for one-half hour. Result, hemolysis trace.

In the first experiment one complete lytic dose does not give complete hemolysis; there is evident inhibition, and this can now be explained on the basis of "Komplementoid Verstopfung." In the second experiment there was very little antibody present in the "decant" serum, but in the presence of complement, icing with untreated corpuscles gave slight hemolysis.

If corpuscles do take up antibody and complement in the cold, it should be possible to demonstrate this action also. For this purpose I have repeated one of Moss' experiments, using the exact proportion given by him and practically the same technique:

Experiment. 1 c.c. "P. Ser." (active) and 0.1 c.c. "P. Corp.," containing as little salt solution as possible, were iced for one-half hour, centrifuged in the cold, and corpuscles washed twice with salt solution at 0° C. They were suspended in 0.75 c.c. salt solution and incubated at 37° C. for one-half hour. Result, hemolysis slight. Complement added (0.25 c.c., "N. H. S.") and temperature maintained at 37° C. for two hours. No increase of hemolysis. Incubated at 0° C. one-half hour, then 37° C. for two hours. No apparent increase of hemolysis.

The result obtained here is different than that given by Moss, who found hemolysis after the addition of complement and without exposure to the cold. In my experiment hemolysis showed after incubating the washed cells, and must have been due to anchored antibody and complement. This is the first positive result of this sort recorded, and it has been possible to duplicate it, although not constantly. The demonstrated dissociability of antibody and complement may be the explanation, although I am inclined to attribute it to the dissociation of complement, either "end-piece" or "mid-piece" from the combination, for it is readily conceivable that prolonged washing of corpuscles even with ice-cold salt solution might liberate end-piece or mid-piece of complement from corpuscle antibody combination. Experiments to determine this point have not been done. Hemolysis in this experiment was slight only, but if we analyze this it is found that we have but two and one-half complete lytic doses of serum (0.4 c.c. "P. Ser." was the minimum complete lytic dose on this day) added to the sediment of eight units of corpuscles. Naturally they are not highly sensitized and no greater hemolytic effect could be expected.

Meyer and Emmerich[8] give an experiment in which a tourniquet was placed about the finger of a hemoglobinuric, and it was immersed in ice water ten nimutes. The blood then taken from the finger was centrifuged and the serum decanted. They do not state whether the corpuscles were washed or not. To the corpuscles was added normal human or guinea-pig serum, and without any further cooling, but merely on incubation, hemolysis resulted. They give this as an example of the union of complement to antibody in the warm, but there is no ground for their conclusion, for the result can be explained on the supposition that complement, as well as antibody, was already bound to the corpuscle "*in vivo*," and the complement added took no part in the reaction. The same result might have been obtained merely on the addition of salt solution without any complement. They do not state, however, that any such control was done.

It has not been possible to explain all of the varying results of experiments found in the literature on any one basis. It is a well-known fact that in cases where the paroxysms have been frequent the "cold-warm" experiment *in vitro* is very apt to be negative. In such cases we may presume a deficiency or absence of complement, and Meyer and Emmerich have shown that such did occur in some of their cases. Even in normal serum it has been frequently shown that there is considerable variation in the complement content from day to day. That absence of antibody occurs seems less likely on account of the difficulty of absorbing all of it even from defibrinated blood, but a deficiency might occur temporarily, although we know nothing as to the rapidity of its formation. That anti-complementary substances can and do occur is well known and Kumagai and Inoue appear to have demonstrated their presence in paroxysmal hemoglobinuria in a number of their cases.

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[8] Loc. cit.

Such a formation could well account for the apparently spontaneous cures that take place from time to time in the course of this disease. Such a remission is shown in the history of this case. No such substance was present, however, during the period of observation. The negative and anomalous results so frequently obtained in test tube experiments with inactive serum are, however, explainable on the ground of complementoid inhibition. To reactivate inactive serum, complement should be added to it and mixed before corpuscles are added. By this technique positive results can be obtained which might otherwise be negative.

7. ETIOLOGY OF PAROXYSMAL HEMOGLOBINURIA. In spite of the work that has been done on paroxysmal hemoglobinuria the basic etiologic factor is still a matter of speculation. Considering the fact that we are apparently dealing with a true autolysin, our general knowledge of the production of such bodies certainly suggests that it may be the result of the rapid and extensive disintegration of homologous or autogenous red blood corpuscles within the body. In the first place, then, concealed and internal hemorrhage would seem to offer the proper predisposing factor. This aspect of the subject was reviewed by Eason in his second paper. Traumatic hemoglobinurias are on record, but while such hemorrhages are common, a resulting hemoglobinuria is extremely rare, is merely temporary and unlike the disease under discussion, in that cold is not a factor in the production of the hemoglobinuria.

Then, again, malaria is a condition that fulfils all the theoretical requirements for the production of a lysin. The conception of hemoglobinuric fever as a malarial manifestation is very earnestly disputed at the present time; in fact, the trend of opinion among authorities on tropical medicine is rather strongly in favor of its independent origin, but even were we to consider hemoglobinuric fever as a malarial manifestation, it would still be impossible to regard malaria as in any way etiologically related to paroxysmal hemoglobinuria, for on clinical grounds they are absolute antitheses as far as the mortality and the relation of climate to attacks is concerned. Moreover, a malarial history is very infrequently elicited in paroxysmal hemoglobinuria, and in spite of very careful experimental search, Barratt and Yorke[9] were not able to demonstrate an autolysin in the serum of cases of malaria or hemoglobinuric fever.

Finally, animal experimentation has been resorted to in an attempt to demonstrate the production of autohemolytic antibodies under ideal conditions. Ehrlich and Morgenroth[10] first studied this subject. They were unable to induce the formation of an autolysin in goats, experimentally, by the intraperitoneal injection of the laked blood of other goats, although an active isolysin developed.

[9] Ann. Trop. Med. and Hyg., 1909, iii, 152, Series T. M.

[10] Berl. klin. Woch., 1900, No. 21, p. 1 and 11.

They did not try the effect of cold on uniting any hypothetical auto-antibody to the red blood corpuscles. They argue that as the injection of an isolysin causes the production of anti-isolysin, so would the production of an autolysin lead to the formation of its antibody, but no such body could be demonstrated. That such an antibody need not develop, however, is abundantly proved by the fact that none has been demonstrated in the serum of hemoglobinurics in spite of the presence of the autolysin. Fejes and Kentzler[11] did succeed in one case of four when they injected rabbits, first with aleuronat solution intraperitoneally, and on the next day injected intraperitoneally 7 c.c. of autogenous blood plus the complete lytic dose of goose serum for this amount of blood. They state that the serum from one rabbit showed a hemolysin that behaved in all respects like that of paroxysmal hemoglobinuria in test tube experiments, but hemoglobin never appeared in the urine when the animal was chilled.

One fact that is particularly prominent is the association of syphilis, either congenital or acquired. Clinical observation of itself is quite impressive. Stempel (1902), quoted by Eason, found 30 per cent. positive in 77 cases in the literature. Of Kumagai and Inoue's 20 cases, 85 per cent. are given as luetic.

With the introduction of the Wassermann reaction these percentages have been still further increased. Of 37 cases now in the literature, including my own, on which the Wassermann reaction is reported, 33 or 90 per cent. are positive. It is, however, essential to bear in mind that this reaction is not specific; that is, is not dependent upon syphilitic antibodies for the complement absorption. This is evidenced by its occurrence in the serum of a large percentage of normal rabbits and in human serum just before and after death, irrespective of cause, by its high percentage in frambesia, leprosy, relapsing fever, and malaria, and particularly by the fact recently reported by Noguchi, but still unpublished, that complement binding rarely took place when he used as antigen his pure culture of treponema pallidum, although the Wassermann reaction, done simultaneously, was positive.

It was in view of these facts that the luetin test was done in the case here reported. Noguchi[12] found his reaction positive in 96 per cent. of 23 hereditary cases and absent in all of 146 controls. As stated above, the reaction was positive in this case. It is, however, too new to warrant our placing absolute dependence upon it at this time.

That syphilitic infection does seem to bear definite relation is further shown by the investigation of Donath and Landsteiner. They examined the serum of 65 cases of general paresis *in vitro* for an autolysin, and found it present in 6 cases, two of which gave very marked reaction and 4 but slight. Using the 2 severe cases, the Ehrlich experiment, by icing a finger, showed hemolysis, but they were not able to induce an attack of hemoglobinuria in either. In 195 other cases of infectious and systemic disease, newgrowths and intoxications, including 28 cases of "lues," they were not able to show the presence of any hemolysin in the serum by their test tube experiments. They were led to suggest that the presence of the hemolysin in the serum is not sufficient of itself to induce the urinary manifestations of the disease and that vasomotor disturbances might be important contributing factors favoring the necessary temperature depression in the exposed tissues. Kumagai and Inoue have also recently reported such examinations for an autohemolysin. Thirty cases that were non-syphilitic by history and Wassermann reaction showed no lysin present. Their 13 cases of secondary syphilis also showed no lysin. Of 35 cases of tertiary and metasyphilitic disease 7 showed the presence of a hemolyzing substance. The Ehrlich experiment was performed on 4 of these, and 1 case gave a typical manifestation of paroxysmal hemoglobinuria. The other 3 responded by an attack which these authors consider a mild form of the disease; that is, albuminuria and urobilinogenuria with typical temperature and leukocyte changes but no hemoglobinuria.

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[11] Zeit. f. klin. Med., lxxi, Heft 3 to 6, p. 191.

[12] Jour. Exp. Med., 1911, xiv, No. 6.

We have, however, at our command one further and perhaps crucial test by which to determine the syphilitic or non-syphilitic nature of this disease, and that is the therapeutic test. Of the older writers, cited by Eason, Murri (1885) reports 2 cases and Kopp (1885) 1 case cured by inunctions of mercury. One of Moss' cases received "salvarsan," and while the Wassermann reaction was still positive at the end of four months, the attacks seemed to be beneficially influenced, although the autolysin was still present in the serum. Strange to say, of all the recent cases in literature not one has been followed to its definite and logical conclusion in this regard; that is, no observations have been made on any case in which, as a result of treatment with mercury and iodide and salvarsan, the Wassermann reaction has become negative. My own case still has a strongly positive reaction four weeks after the third injection of "salvarsan." I hope to be able to report observations later after the Wassermann has become negative.

SUMMARY. The observations here reported are based upon experiments with the blood of a typical case of paroxysmal hemoglobinuria.

The serum contains a complex hemolysin capable of dissolving the red blood corpuscles of the individual himself or other individuals by means of the cold-warm experiment. Positive results were obtained in all of the twelve examinations made at different times.

Auto-antibody is absorbed from serum in the absence of complement, and on elevation of temperature is more or less completely but slowly dissociated.

Complement is absorbed from active serum on exposure to corpuscle in the cold. It will join with antibody after the latter has united with corpuscle, but this union takes place solely under the influence of cold.

Corpuscles sensitized with inactive serum show but slight hemolysis on the addition of complement as a result of complementoid inhibition.

As a result of clinical observation, the Wassermann reaction, the luetin test, and the serological studies in metasyphilitic disease, it seems safe to say that syphilis is the most important, possibly the only, etiologic factor in paroxysmal hemoglobinuria, but there are as yet no observations on the presence or absence of the hemolysin after the disappearance of the Wassermann reaction as a result of syphilitic treatment.

A CASE OF DELAYED DEVELOPMENT IN A BOY TREATED WITH THYMUS GLAND.[1]

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The physiology of the thymus gland is very imperfectly understood. No one of the ductless glands appears to have a more active part in the economy, especially in the younger years of life, and yet its complete removal is not accompanied by those striking manifestations which follow removal of the thyroid or adrenal. It cannot be concluded, however, that because the removal of a gland is not followed by an acute death, it plays no important part in nutrition. The evidence afforded by removal of the sexual glands is proof on this point.

The laboratory investigations are not altogether in accord. Certain points, however, seem to be fairly well established. The thymus remains in an active condition during the presexual life of an animal, but with the onset of sexual maturity it undergoes a retrogression which finally amounts to an almost complete atrophy. The involution of the thymus appears to be a more rapid process when the sexual glands become mature. When castration is performed before puberty the thymus does not show the same degree

[1] Read before the American Pediatric Society, Hot Springs, Va., May 30, 1912.